IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of)
)
Guy GOROCHOV et al)
)
Serial No.:10/791,814)
)
Filed: March 4, 2004)
)
For: METHODS FOR CONSTRUCTION AND SCREENING OF LIBRARIES OF CHEMOKINE VARIANTS

Conf.:3637

Group Art Unit: 1639

Examiner: T.D. Wessendorf

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DECLARATION CONCERNING INVENTORSHIP
BY ALL FIVE INVENTORS

Commissioner of Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

We, Guy Gorochov, Oliver Hartley, Karim Dorgham, Patrice Debre and Robin Offord, do hereby declare as follows:

[1] That we have read and understood the specification and claims of the subject application entitled ":METHODS FOR CONSTRUCTION AND SCREENING OF LIBRARIES OF CHEMOKINE VARIANTS", the Declaration of which was executed in March and April 2004;

[2] That we are the co-inventors of the subject application;

[3] That the five inventors, namely Guy Gorochov, Oliver Hartley, Karim Dorgham, Patrice Debre and Robin Offord were listed as co-authors of the Abstract entitled "A Potent Anti-HIV Compound Produced Through Knowledge-based Design of a Phage Display Selection Strategy" , *Institute of Human Virology 2000 Meeting Abstracts" Journal of Human Virology*, Volume 3, Number 5, September/October 2000.
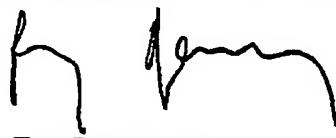
[4] That Danielle Perez-Bercoff, Annie David, Hubert Gaertner, and Gianfranco Pancino, the other authors of the Abstract identified in paragraph [3] above merely carried out assignments and worked under the supervision and control of one or all of the inventors of the subject application and were listed as co-authors in order to receive credit for having collaborated in the research program under the direction and control of the five inventors; and

[5] That only, Guy Gorochov, Oliver Hartley, Karim Dorgham, Patrice Debre and Robin Offord are the true inventors of the subject application.

[6] We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Date ___21ᵗʰ of Dec 2005___   _____
                                        Guy Gorochov,

Date _____   _____
                                        Oliver Hartley

Date ___21 -XII - 2005___   _____
                                        Karim Dorgham

Date ___21.12.2005___   _____
                                        Patrice Debre

Date _____   _____
                                        Robin Offord

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Date _____

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Guy Gorochov,

Date _____

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Oliver Hartley

Date _____

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Karim Dorgham

Date _____

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Patrice Debre

Date   20 Dec 2005_____

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Robin Offord

NCBI

MMDB
Structure Summary
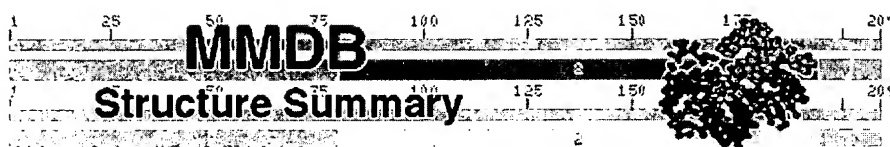
PubMed   BLAST   Structure   Taxonomy   OMIM   **Help?**

Description: Phosphomannose Isomerase.

Deposition: A.Cleasby, T.Skarzynski, A.Wonacott, G.J.Davies, R.E.Hubbard, A.E.Proudfoot, M.A.Payton & A.R.Bernard, 3-Apr-96

Taxonomy: Candida albicans

Reference: PubMed   **MMDB:** 4973   **PDB:** 1PMI

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Protein	Chain
3d Domains	1 2 3 1
CDs	PMI_typeI

(scale: 1, 50, 100, 150, 200, 250, 300, 350)
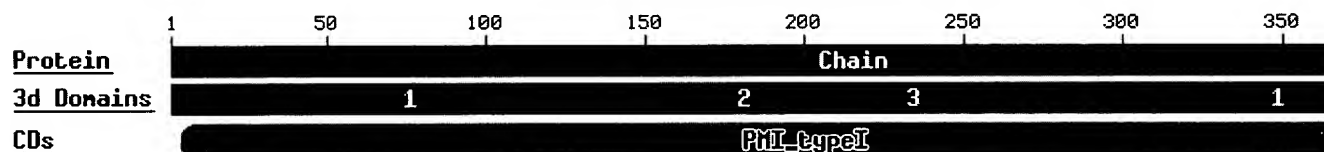
Citing MMDB: Chen J, Anderson JB, DeWeese-Scott C, Fedorova ND, Geer LY, He S, Hurwitz DI, Jackson JD, ⌣ Lanczycki CJ, Liebert CA, Liu C, Madej T, Marchler-Bauer A, Marchler GH, Mazumder R, Nikolskaya AN, Rao BS Shoemaker BA, Simonyan V, Song JS, Thiessen PA, Vasudevan S, Wang Y, Yamashita RA, Yin JJ, Bryant SH, " structure database", **Nucleic Acids Res. 2003 Jan; 31(1):** 474-7.

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Review Article

BIOLOGY OF THE RANTES/SIS CYTOKINE FAMILY

Thomas J. Schall

The field of cytokine research has undergone a quiet but vigorous expansion in the last half decade. Advances in the understanding of the activities of the "traditional" immune cytokines such as the tumor necrosis factors (TNFs), the interferons (IFNs), the interleukins (ILs), and the colony stimulating factors (CSFs), have been enabled in part by the elucidation of the nature of the specific receptors for many of these molecules, and efforts are ongoing to dissect their signal transduction mechanisms. But the last five years have also seen the opening of a new field in immunobiology. During this time an entirely new class of cytokines has been discovered and characterized. This emerging superfamily of small secreted factors shows a degree of relatedness not before seen with traditional immune cytokines, and its study promises the potential of key insights into a broad range of areas in immune regulation and disease states.

Members of this new class of cytokines, which has been referred to as the platelet factor 4 (PF4) superfamily,[1] are related by predicted primary structural similarities and by the conservation of a four cysteine motif. The superfamily is bipartite, with the two branches being classified according to the position of the first two cysteines in the conserved motif. The "C-X-C" branch, which includes such molecules as PF4 and interleukin-8 (IL-8), is characterized by the separation of the first two cysteines in the primary structure by an intervening amino acid (Fig. 1).[2-4] In the "C-C" branch these two cysteines are directly adjacent. The C-X-C branch of of this superfamily claims a longer and larger history of experimental investigation, with PF4, its prototype, being first described in 1955;[5] its amino acid sequence was detailed in 1977.[6] More recently IL-8 and its proinflammatory activities have received a great deal of attention. Indeed, many of the molecules in the C-X-C branch appear to have proinflammatory properties, mainly through their actions on neutrophils. The PF4 superfamily has been covered generally in a recent

review,[1] and IL-8 and related proteins have been specifically examined in a number of recent papers.[7-9]

This review will concentrate primarily on the C-C branch of the PF4 superfamily. It will describe the general properties of these molecules and detail their possible roles in physiological and pathological processes, while at the same time attempting to put their investigative histories into perspective with a view towards simplifying some of the apparent complexity of this family. The term "RANTES/SIS" has been used for the proteins of human origin in this family and, more for convenience than by convention, this designation will be generally used here. Rapid expansion in this field, as well as independent reports of similar or identical molecules and the independent characterization of proteins of both mouse and human origin has resulted in non-standard and sometimes complex nomenclature (Table 1). Efforts are ongoing to solve this problem, but for the purposes of this report, identical or nearly identical molecules of all species will be classified under a single name as described below, with appropriate references to their alternative designations.
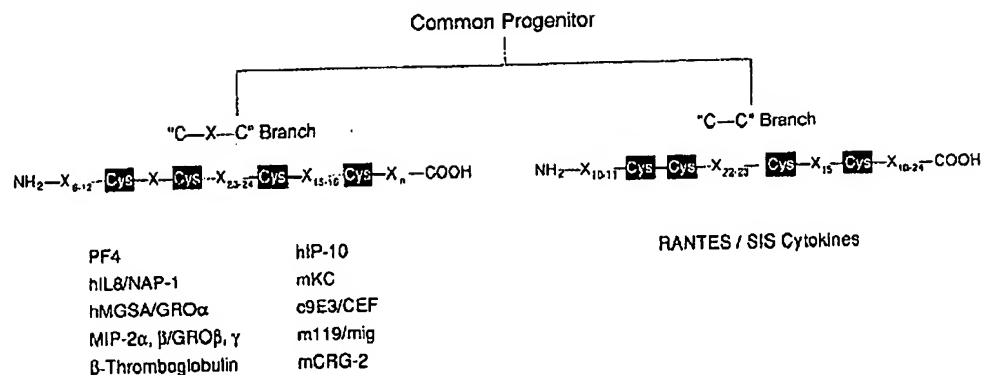
GENERAL CHARACTERISTICS OF RANTES/SIS CYTOKINES

The human RANTES/SIS cytokine family comprises at least six distinct molecules: RANTES, I-309, monocyte chemotactic protein-1 (MCP-1), HC14, and the macrophage inflammatory proteins HuMIP-1α and HuMIP-1β. Other names for these molecules, representing either independently isolated clones or slightly variant molecules, and the identity of their mouse homologs are listed in Table 1. Most of the molecules in this family were first identified by molecular cloning techniques as cDNAs encoding proteins of unknown function. It is only now that many of their biological properties are coming to light. A schematic representation of the approaches used to identify these cytokines, along with the molecules identified by each technique, is shown in Fig. 2. Many of the techniques employed to identify RANTES/SIS cDNAs exploited the intense, rapid induction of the mRNAs that encode some of these cytokines in immune cells. Typically, T lymphocytes or peripheral blood mononuclear cells (PBMC) have been stimulated mitogenically (with lectins or

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Common Progenitor

"C—X—C" Branch

$NH_2—X_{6-12}—$Cys$—X—$Cys$—X_{23-24}—$Cys$—X_{15-16}—$Cys$—X_n—COOH$

PF4	hIP-10
hIL8/NAP-1	mKC
hMGSA/GROα	c9E3/CEF
MIP-2α, β/GROβ, γ	m119/mig
β-Thromboglobulin	mCRG-2

"C—C" Branch

$NH_2—X_{10-17}—$Cys$—$Cys$—X_{22-23}—$Cys$—X_{15}—$Cys$—X_{10-24}—COOH$

RANTES / SIS Cytokines

Figure 1. Organization of the platelet factor 4 superfamily.

Diagrammatic representation showing the relationship of the protein structures of "C-X-C" and "C-C" cytokines in the superfamily. RANTES/SIS cytokines comprise the C-C branch, platelet factor 4 and related molecules as listed form the C-X-C branch (for review and references, see references 1-4). Cysteine residues are boxed and the number of intervening amino acids between them (X) is denoted by the subscript. The number of carboxyl-terminal amino acid residues for the C-X-C branch (X_n) ranges from 15 to 53. Lower case prefixes for molecules listed denote species: h = human, m = mouse, c = chicken. Molecules without prefixes indicate multiple species homologs have been identified.

phorbol esters) or antigenically and cDNA libraries constructed from RNA harvested from these cells shortly after stimulation. Most of the various cDNA clones encoding HuMIP proteins have been discovered in this way, and murine JE (the homolog of human MCP-1) was originally isolated from serum-stimulated fibroblasts. By design, a hallmark feature of the molecules identified in this fashion is their marked *inducibility*. In fact, in their induced states, the mRNA for these molecules can represent 1% of the total poly(A)' RNA in the parent cells.[17,19,13] In contrast, most are not expressed in the unstimulated cell. This property, and the small size of the predicted secreted gene product, led to the early use of the "SIS" designation (Small Inducible Secreted)[13] for molecules of this class, as well as their inclusion in the "SIG" class (Small Inducible Genes).[40] Though inducibility is a feature of most of these genes, it is not a universal property. For example, RANTES mRNA is actually *reduced* after activation of

differentiated "functional" T cell lines, even with the concomitant induction of some of the other members of the family. Hence the term "RANTES (Regulated on Activation, Normal T Expressed and Secreted)/SIS" may be more accurate, and reflects the designation used in two of the earliest reports recognizing a relationship among these molecules.[10,13]

Biochemical approaches, as well as studies in cellular immunology, have also led to the identification of molecules in this family (Fig. 2). The identification of monocyte chemotactic factors produced by lymphocytes and tumors, for instance, led to the eventual purification and cloning of one of these factors, MCP-1, which was the human homolog of the earlier identified mouse JE. Similarly, protein biochemical characterizations of potential inflammatory factors released from stimulated murine macrophages led to the identification of the murine MIP-1 molecules.

All RANTES/SIS cytokines share a typical primary

Table 1. C-C cytokines.

Roster of RANTES/SIS cytokines, alternative names, mouse homologs, and variant clones. References describing isolation of molecules or use of alternative designations are listed in parentheses.

RANTES/SIS Cytokines	RANTES (10)	I-309 (11)	HuMIP-1α	HuMIP-1β (22)	MCP-1 (29)	HC14 (37)
Alternative human names and variant clones	hSISδ (13)	hSISε (13)	pLD78, LD78 (14) hSISα, hSISβ (13) pAT464 (15) GOS19 (16)	hH400, hSISα (13) Act-2 (23) pAT744 (15) G-26 (11) HC21 (24) HIMAP (25,26) MAD-5 (27)	MCAF (30,31) hJE (32) LDCF, GDCF (33-35) MCP-1α, -1β (36) HCll (37) TSG-1 (38)	—
Mouse homologs and alternative names; variant clones	—	TCA3 (12) P500, SISε (13)	TY5, SISα, SISβ (17,13) MIP-1, MIP-1α (18,19) SCl (20) L2G25B (21)	H400, SISγ (13) MIP-1, MIP-1β (18,28)	JE (39)	—

Induction–Specific/ Differential Hybridization

pLD78	TCA3
GOS19	P500
Act–2	JE
pAT464	TY5
pAT744	H400
MAD–5	L2G25B
TSG–1	

Subtracted Libraries/ Subtracted Probes

RANTES	HC21
I–309	HC11
G–26	HC14

Biochemical Purification of Inflammatory Factors

MIP–1

MIP–1α   MIP–1β

Lymphocyte & Tumor Produced Chemotactic Agents

LDCF	MCAF
GDCF	MCP–1
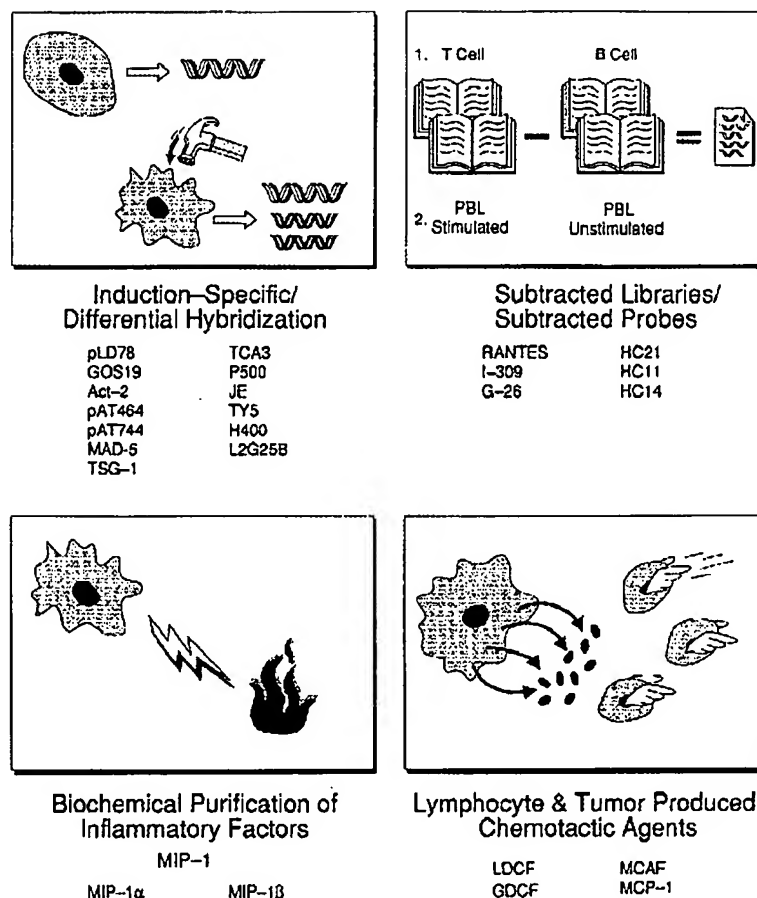
Figure 2.   Isolation of RANTES/SIS cytokines.

Schematic representation of methods by which RANTES/SIS and related cDNAs were obtained. Names of independent clones and variants are listed beneath the appropriate category. Human clones are listed to the left and mouse clones to the right in the differential hybridization category. Other categories list either all human or all mouse molecules as described in the text.

structure: a cleaved signal peptide of about 20 to 24 amino acids and a mature secreted protein of 68 to 76 amino acids. Each of four cysteines is absolutely conserved in the six molecules of the family, as are a number of other residues (shaded boxes and areas, Fig. 3A). Other amino acids, though not absolutely conserved, appear in a majority of the cytokines in the family (Fig. 3A). The four conserved cysteines probably exist as disulfide bridges, as has been shown to be the case with β-thromboglobulin (βTG),[44] PF4,[42] and IL-8[43,44] in the C-X-C branch and MCP-1[36] in the RANTES/SIS branch. In this arrangement, disulfide bridges link the first cysteine to the third, and the second to the fourth, resulting in a secondary structure with two loops. The intact looped domains are probably essential to the functions of these molecules, as suggested by the loss of function of IL-8 after reduction and alkylation.[44] Since it appears that proteins of the C-C class will not bind receptors specific for C-X-C proteins (references 45 and 46, and discussed below), it is likely that binding specificity is a function of differences in primary structure rather than of the overall array of the looped domains.

The primary sequences of the RANTES/SIS cytokines predict secreted molecules with masses of 7,800 to 8,700 daltons. Their actual biochemical behaviors are somewhat more diverse than this. RANTES/SIS cDNAs, when expressed in mammalian cells, encode secreted molecules with apparent molecular masses ranging from 8,000 to 18,000 daltons (Fig. 3B). Only RANTES and HuMIP-1α migrate as predicted from their primary structure. HuMIP-1β, though 70% identical to HuMIP-1α in primary structure, migrates as a much more diffuse mass ranging from 8 to 12 kD. MCP-1 exhibits at least two bands of approximately 10 and 14 kD, and I-309 (kindly provided by M. Miller and M. Krangel, Duke University) appears in a number of forms: a protein doublet of 15 to 16 kD and a minor band corresponding to an 8-kD core protein. The reasons for these diverse behaviors are not wholly known, but, as discussed below, these related cytokines display an unexpected array of behaviors and properties.

MIP-1 Proteins

The biochemistry and investigative history of the two proteins of which "macrophage inflammatory protein 1" (MIP-1) is composed make it useful to discuss them jointly. The name MIP-1 reflects the fact that these two proteins were originally copurified from endotoxin-stimulated mouse macrophages and were defined as a single protein.[18] Many of the biological activities of what we now know to be two closely related
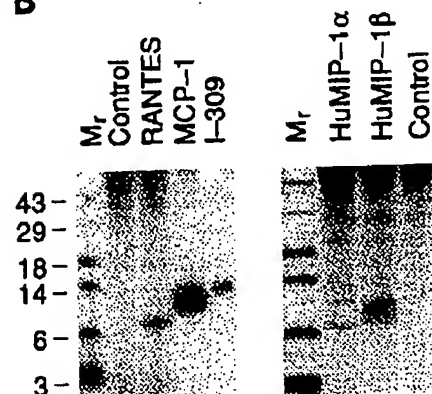
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Figure 3. Structures of RANTES/SIS cytokines.

(A) Alignment of predicted amino acid sequences of the six distinct human RANTES/SIS cytokines. Conserved four cysteine motif is shaded and boxed; other absolutely conserved residues are shaded. Residues conserved in a majority of members are in boldface. Numbering is relative to position in RANTES. Single letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (B) SDS-PAGE analysis of recombinant RANTES/SIS cytokines. Tris tricene gel (16%) fractionation of biosynthetically labeled supernatants from human embryonic kidney 293 cells transfected with a control expression plasmid lacking a cDNA insert, RANTES and MCP-1 expression plasmids, and immunoprecipitated I-309 protein (left panel). Right panel is a comparison of control, HuMIP-1α, and HuMIP-1β labeled supernatants. Left-hand lane in both panels is migration of ^{14}C M, standards (scale × 10^{-3}). Transfections were carried out as described.[67]

but distinct proteins, MIP-1α and MIP-1β (HuMIP-1α and HuMIP-1β in humans), were subsequently explored with the murine MIP-1 protein preparation and some of these activities may require the presence of both proteins.

Prior to the description of MIP-1, a human cDNA clone designated pLD78, which encoded a protein of unknown function, was isolated from human tonsillar lymphocytes that had been stimulated with mitogen and phorbol ester.[14] This clone, the first described cDNA for any of the RANTES/SIS cytokines, was found to be highly inducible: its mRNA expression rapidly increased 30-fold in lymphocytes after stimulation with phytohemagglutinin (PHA) and the phorbol ester 12-O-tetradecanoyl phorbol acetate (TPA, reference 14). The same report described a genomic clone, LD78, which appeared to be highly related to, but distinct from, pLD78. Later studies have shown that these two clones represent two distinct but highly related genes. The genomic organization of the RANTES/SIS cytokines is discussed below.

The first isolated murine homolog for LD78 was TY5, a cDNA obtained from activated T helper cells and identified in a 1986 paper,[17] but whose sequence was not reported until much later.[13] In 1988 Cerami

and co-workers identified MIP-1 while purifying TNF and an inhibitor of friend erythroleukemia cell differentiation from endotoxin-stimulated mouse macrophages.[18] MIP-1 was described as an acidic, heparin-binding protein that resolved as a doublet of approximately 8,000 daltons on SDS-PAGE.[18] The MIP-1 protein preparation caused footpad inflammation characterized by an infiltration of neutrophils in endotoxin-resistant mice, as well as mild neutrophil chemokinesis and low amounts of H_2O_2 release from neutrophils in vitro. The in vitro activities were found to be maximal at relatively high concentrations of MIP-1 (≥ 1 μg/ml), and these high concentrations were thought to be necessary because of the propensity of MIP-1 to form large aggregates.[18,47] It has therefore been difficult to assign activities for MIP-1 on a molar basis. It is also possible that at high concentrations MIP-1 might be acting on neutrophils via a receptor for a related molecule, such as IL-8, and these activities might not reflect a direct or major physiological role of MIP-1. MIP-1 has been shown to have other unique properties: it is the first known endogenous pyrogen that works via a prostaglandin-independent pathway,[48] and it can synergize with the hematopoietic growth factors granulocyte-macrophage CSF (GM-CSF) or

macrophage CSF (M-CSF) to enhance granulocyte-macrophage colony formation.[49]

Partial NH$_2$-terminal sequencing of MIP-1 revealed a major sequence with minor residues occurring consistently at the same positions.[18] Further biochemical analysis showed that MIP-1 was composed of two distinct but highly related proteins, designated MIP-1α and MIP-1β. Cloning studies confirmed that murine MIP-1α and MIP-1β were encoded by separate genes.[19,28] The mature secreted forms of murine MIP-1α and MIP-1β, as well as their human homologs, are nearly 70% identical.

An almost bewildering number of cDNAs encoding the MIP-1 proteins and their human homologs (which for the sake of convenience will all be categorized as either "HuMIP-1α" or "HuMIP-1β") have been independently obtained by a number of groups using a variety of methods (Table 1 and Fig. 2). In addition to TY5 (a MIP-1α equivalent), Zurawski and colleagues obtained both mouse and human H400 (MIP-1β equivalents) and murine P500 (a variant of TCA3/I-309, see below) by differential hybridization.[13] All of these cDNAs represented very abundant mRNAs [0.5 to 1% of total poly(A)$^+$mRNA] induced in stimulated T helper cells.[13] Another murine clone identical to MIP-1α/TY5, designated L2G25B, was obtained by differential screening.[21]

The human MIP-1α (HuMIP-1α) cDNAs that have been independently cloned include pLD78, pAT464 (obtained from PBL stimulated with PHA and TPA, reference 15), and GOS19, isolated from PBMC as a gene expressed in the G$_0$ to G$_1$ switch induced by lectin and cyclohexamide treatment.[16]

The existence of several independently isolated clones encoding HuMIP-1β also causes some confusion in this family. There are at least seven independent reports of HuMIP-1β cDNAs, many of which vary slightly in the proteins they encode: Act-2, identified by differential screening of an activated T-cell library;[23]

pAT 744, isolated from stimulated PBL along with pAT 464;[15] G-26, identified jointly with another RANTES/SIS cytokine, I-309 (see below) from activated PBMC;[11] HC21, cloned from PBL stimulated with anti-CD2 mAb;[24] and finally MAD-5α and MAD-5β, which were cloned from adherence-induced monocytes.[27] MAD-5β is an exact, albeit truncated, match of Act-2, and MAD-5α exhibits a 115-bp deletion after the leader sequence. This deletion results in a completely frame-shifted open reading frame encoding a putative mature peptide of 28 amino acids. It is not clear whether MAD-5α represents a mature mRNA but Sporn et al. suggest that MAD-5α is an active transcript.[27]

Many of these HuMIP-1β cDNAs encode slightly variant proteins, being between 94% and 98% identical. A cDNA clone isolated in this laboratory, formerly referred to as HIMAP[25,26] and now simply referred to as HuMIP-1β, is shown aligned with other HuMIP-1β–encoding cDNAs in Fig. 4. The functional significance of this "family within a family" is not known, but recent evidence suggests that there are multiple nonallelic forms of both HuMIP-1α and HuMIP-1β genes,[16,50,51] and this could account for the microvariability of these cytokines.

Expression of mRNAs for human and mouse MIP-1α and -1β suggest that they are mostly restricted to hematopoietic cells including B and T lymphocytes, mast cells, and myeloid cell lines.[15,23,27,52,53] Recent evidence also suggests that HuMIP-1α is expressed in fibroblasts under certain circumstances (reference 51 and unpublished observations) and in a number a fresh hematopoietic tumors and cell lines.[54]

Biological function data on the MIP-1 proteins has mostly been obtained from mouse systems and has suggested that these molecules have proinflammatory properties. As the mouse data were obtained mainly with the MIP-1 protein preparation, it is not clear whether all of the activities of murine MIP-1α and

Figure 4. Variation among predicted proteins from independent HuMIP-1β clones.

Predicted primary sequence comparison of HuMIP-1β isolated in this laboratory with independently isolated clones Act-2, pAT 744, hH400, and G-26. The mature protein begins at Alanine 24 as described for Act-2.[23] Amino acid abbreviations as in Fig. 3A. Dashes represent identical residues, differences are listed beneath the HuMIP-1β sequence. Deletions are represented by (*).

SIGNAL SEQUENCE

HuMIP1 β:	M	K	L	C	V	T	V	L	S	L	L	V	L	V	A	A	F	C	S	L	A	L	S
Act-2:	-	-	-	-	-	-	-	-	-	-	M	-	-	-	-	-	-	P	-	-	-
pAT 744:	-	-	-	-	-	-	-	-	-	-	M	-	-	-	-	-	-	-	-	-	-
hH400:	-	-	-	-	-	-	-	-	-	-	M	-	-	-	-	-	-	P	-	-	-
G-26:	-	-	-	-	-	-	-	-	-	-	M	-	-	-	-	-	-	P	-	-	-

MATURE PROTEIN

HuMIP1 β:	A	P	M	G	S	D	P	P	T	A	C	C	F	S	Y	T	A	R	K	L	P	H	N	F	V	V	D	Y	Y	E	T	S	S	L	C	S
Act-2:	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pAT 744:	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
hH400:	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	E	A	S	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G-26:	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	I	-	-	-

HuMIP1 β:	Q	P	A	V	V	F	Q	T	K	R	G	K	Q	V	C	A	D	P	S	E	S	W	V	Q	E	Y	V	Y	D	L	E	L	N
Act-2:	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pAT 744:	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
hH400:	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G-26:	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-

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MIP-1β are separable. There have been as yet few reports of data obtained with the murine recombinant MIP-1α and MIP-1β, proteins. One finding suggests that MIP-1α, but not MIP-1β, is a specific inhibitor of hematopoietic stem cell proliferation.[20] To date there is no published information on the activities of the human proteins. The disparate biochemical nature of the mature human MIP-1α and MIP-1β proteins (Fig. 2B) hints that the biological properties of these cytokines could be different. This is supported by studies in this laboratory, which indicate that HuMIP-1α and HuMIP-1β specifically target and attract separate subpopulations of lymphocytes, and thus may be important in lymphocyte trafficking.[22] The relevance of these and other findings regarding the activities of the MIP-1 proteins proteins is discussed below.

MCP-1

In 1970, Ward and co-workers provided early experimental evidence for the existence of specific lymphocyte-derived monocyte chemotactic factors.[55] In that study lymphocytes from sensitized guinea pigs, when stimulated in vitro with the sensitizing antigen, produced a mononuclear cell chemotactic factor. Shortly thereafter, Snyderman and Altman and colleagues obtained a partially purified monocyte chemotactic factor with a mass of ~12.5 kD from antigen-induced lymphocytes, which they designated LDCF.[33]

Nearly ten years later Stiles and co-workers reported that gene "JE" could be massively induced in murine fibroblasts by platelet-derived growth factor (PDGF),[56] and this gene was subsequently characterized by Rollins, et al.[39] Murine JE encoded a small, secreted glycoprotein of unknown function with a mass of ~25 kD. Two groups subsequently purified a human monocyte-specific attracting factor, which they postulated to be the same as LDCF, from a glioma cell line[34,35] and a monocytic cell line.[30] This factor, which in this report is simply referred to as monocyte chemotactic protein-1 (MCP-1, for reviews see references 8 and 36), was originally designated glioma-derived chemotactic factor (GDCF) by Yoshimura et al.,[35] and monocyte chemotactic and activating factor (MCAF) by Matsushima et al.[30] Subsequent cloning of the cDNA for MCP-1 showed it to be highly similar to murine JE.[29,31,32,39] The MCP-1 protein is 62% identical to murine JE in a region of 68 shared N-terminal residues, but murine JE extends an additional 49 residues to elongate the carboxyl portion of the molecule. Though it is widely accepted that JE and MCP-1 are species homologs, the significance of the predicted protein differences is not clear.

Human MCP-1 is a basic peptide of 76 residues with a predicted molecular mass of 8,700 daltons. It behaves on SDS-PAGE, however, as multiple species with masses ranging from 8 to 18 kD. Leonard and

Yoshimura have reported two molecular species of 15 and 13 kD, which they designate MCP-1α and MCP-1β, respectively.[36] The source of the apparent mass difference between MCP-1α and MCP-1β is not due to differences in amino acid composition, and both forms are secreted by cells in the presence of inhibitors of N-glycosylation.[36] Rollins et al. report the secretion of three major forms of recombinant MCP-1 (which they designate human JE) of Mr 15,500, 15,000, and 13,000 from tunicamycin-treated cells.[32] Data from Jiang et al. suggest that differences in the processing of O-linked carbohydrates account for the heterogeneity of MCP-1 produced by different cell types.[57] In addition, an N-terminally processed form of MCP-1 has been reported.[58]

MCP-1 is inducibly expressed mainly in monocytes, endothelial cells, and fibroblasts.[36,59,60] Optimal expression by T lymphocytes seems to require the presence of monocytes. Studies in this laboratory have not detected MCP-1 expression in several T cell lines stimulated by alloantigen in the absence of monocytes, or in a number of T cell clones stimulated with the superantigen Staph enterotoxin B (SEB) or anti-CD3 antibody in the presence of IL-2. Certain transformed cell lines and sarcomas, including the glioma line from which it was purified, seem to constitutively express MCP-1, but normal fibroblasts, endothelial cells, and monocytes require IL-1, TNF, or lipopolysaccharide (LPS) treatment.[36,59-61] MCP-1 gene expression has also been detected in human retinal pigment epithelium cells after IL-1 and TNF stimulation[62] and in epidermal keratinocytes,[63] and Valente et al. report the production of an MCP-1–like protein in baboon arterial smooth muscle cells.[64] The possible significance of MCP-1 production by smooth muscle cells and tumor cells is discussed below.

HC14

A subtracted library made from PBMC stimulated with an anti-CD2 monoclonal antibody was the source of two cDNA clones, HC11 and HC14.[37] Since it has been shown that these sequences are transcribed predominantly in peripheral blood non-lymphocytes, it has been postulated that these genes are induced in monocytes in response to IFN-γ produced by anti-CD2-stimulated T cells.[37]

HC11 is identical to MCP-1 but, interestingly, the sequence reported for HC14 has not previously been described and is 62% identical to MCP-1.[37] This degree of identity approaches that exhibited by the MIP-1 proteins. Since MCP-1 and HC14 are so highly related, and since neither appears to be highly expressed in lymphocytes, these two molecules may be functionally and evolutionarily linked in much the same fashion as the MIP-1 proteins. No biochemical or functional characterizations of HC14 have yet been published,

but future studies should be of considerable utility in evaluating whether HC14 is a functional "MCP-2."

I-309

Burd et al., using subtractive hybridization, identified a gene massively induced in T cells following antigen or concanavilin A (Con A) stimulation.[12] This gene, TCA3, encodes an mRNA that represents as much as 1% of the total poly(A)$^+$ RNA fraction in T cells after Con A treatment. Detection of the human homolog of TCA3 was elusive until the report of the discovery of I-309, a cDNA originally isolated from a γδ T cell receptor (TCR)-positive T cell line.[11] I-309 and TCA3 are only 42% identical in amino acid sequence, but they share an additional pair of conserved cysteines and have marked nucleotide sequence homology in 5' flanking regions.[11] This 5' region includes conserved motifs for an NF-κB–binding sequence, an E1A enhancer core sequence, and a binding site for the macrophage and lymphocyte-specific transcription factor PU.1 (see below).

I-309 mRNA expression is found in a variety of human T cells and in Jurkat cells, but, unlike MCP-1 mRNA expression, is not found in activated endothelial cells or fibroblasts.[11] Similarly, TCA3 mRNA is induced in all subsets of murine T cells tested and in mast cells, but is not present in resting T cells.[12,53] In PBMC, I-309 is not induced by PHA or anti-CD3 stimulation,[11] and there have been no reports of I-309 or TCA3 mRNA expression by monocytic cell types.

Recombinant I-309 expressed in mammalian cells appears as secreted multiple molecular species ranging from 8 to 16 kD (Fig. 3B and reference 11). The murine recombinant TCA3 protein is an N-glycosylated molecule of 16 kD, with a core protein of 8 kD.[65] Though there are no reports yet on the biological activities of I-309, partially purified TCA3 has been shown to cause footpad swelling in mice, suggesting a role for this cytokine in inflammation.[65]

The murine cDNA clone P500[13] probably represents a splice variant of TCA3.[66] P500 is identical to TCA3 except that it contains an insert of 99 bp in the C-terminal coding region. This insert results in an altered carboxyl terminus of the predicted P500 protein relative to TCA3. The presumed mature P500 protein would be 62 amino acids in length rather than 69 for TCA3. P500 would also lack the last of the canonical four cysteines in the conserved family motif, as well as the site for N-glycosylation thought to be functionally linked to a glycan in TCA3.

The genomic loci for I-309 and TCA3 have been characterized.[66] Genomic sequencing of the TCA3 gene reveals that it carries two distinct splice acceptor sites in the second intron. The utilization of the alternative sites could result in different transcripts with distinct third exons. The 99-bp insert present in

P500 represents an insertion immediately 5' to the start of exon 3 of the TCA3 transcript.[66] Hybridization studies using a cDNA probe corresponding to a portion of the 99-bp insertion indicate the presence of approximately one P500 variant for every 12 TCA3 clones in a T-cell library.[13] Alternative splicing of the human I-309 transcript has not been described, but I-309 and TCA3 exhibit a perfectly conserved stretch of 14 nucleotides that span the P500 alternative splice site[66] in the second intron. The overall nucleotide homology elsewhere in that intron is only 36%. Thus, alternative mature mRNA transcripts may exist for both TCA3 and I-309 and encode molecules of as yet unknown functions. The resulting proteins, if translated, would clearly not share the typical two disulfide-bridged structure proposed for all other members of the RANTES/SIS family.

RANTES

Of the cytokines in the family, RANTES is the only one whose transcription is not rapidly induced in hematopoietic cells. In fact, in fully differentiated, functional cultured T cells, the expression RANTES is markedly *reduced* after cellular activation with antigen.[10]

RANTES cDNA was originally obtained from a subtracted cDNA library[10] made from RNA isolated from a functional, non-transformed T cell line 3 to 5 days after antigen stimulation, from which was subtracted RNA from a B lymphoblastoid line. The use of these mRNAs biased the representation of genes in the subtracted library to T cell–specific transcripts present long after the initial burst of early activation transcription. The genes present might also represent those found in normal, but not transformed, lymphoid phenotypes. This idea is supported by the fact that none of the genes isolated from this library, including RANTES, is induced in T cells or PBMC soon after cellular stimulation, shows early activation transcription profiles in T cell lines, or appears to be transcribed by any T cell tumor line tested.[10] The lack of rapid induction in PBMC and, particularly, the unusual reduction of mRNA in stimulated T cell lines may explain why RANTES is unusual in this family in not having been independently isolated by a number of groups (Table 1).

RANTES protein is a highly basic polypeptide (pI ~ 9.5) expressed as a recombinant 8-kD molecule in a mammalian expression system (Fig. 3B and reference 67). It does not appear to be glycosylated. RANTES has been shown to be a chemoattractant for peripheral blood monocytes, and it exhibits the unusual ability to selectively attract T cells of the CD4$^+$/CD45RO$^+$ phenotype in vitro, while not affecting T cells of other phenotypes.[67] The possible significance of

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these attractant properties is examined more thoroughly below.

MOLECULAR GENETICS OF THE RANTES/SIS CYTOKINES

Gene Structure and Chromosomal Localization

Amino acid and predicted structural similarities suggest that RANTES/SIS cytokines, and the superfamily to which they belong, share a common evolutionary history. Molecular evidence is now accumulating to support this view. The gene structures for HuMIP-1α, -1β, MCP-1, and I-309, as well as some of their mouse homologs, have been characterized. All exhibit a three exon/two intron structure, where the first exon contains the 5′ untranslated sequence and coding nucleotides

for the leader peptide, the second exon encodes the N-terminal half of the mature protein, and the third exon consists of carboxyl region–coding nucleotides and the 3′ untranslated region (Fig. 5A and references 16, 50, 66, 68). The fine structure of some of these genes has been mapped, revealing highly conserved intron/exon junctions among these genes. The position of the second intron in all of the molecules is precisely maintained, splitting the codon of a conserved isoleucine residue (Fig. 5B). Similarities in numbers of exons and splice junctions are also conserved between mouse and man (Fig. 5b and references 66, 68, 69). Furthermore, the structure of the genes for the RANTES/SIS cytokines is also very similar to that seen in the C-X-C branch of the PF4 superfamily. The splice junctions between C-C and C-X-C genes are conserved,[66,68]
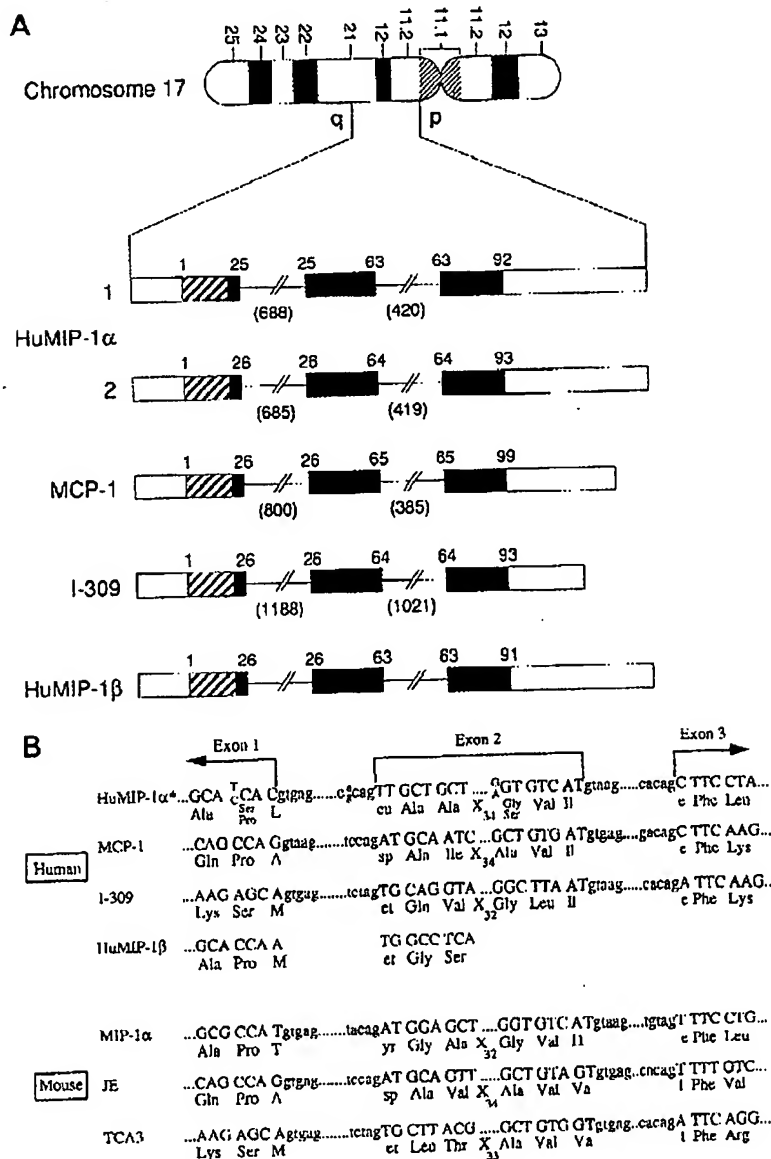
Figure 5. Chromosomal organization of RANTES/SIS cytokine genes.

(A) Intron/exon structure of genes encoding HuMIP-1α (two genes, 1 and 2, as described in references 16, 50, and 51), MCP-1,[36,71] I-309,[66] and HuMIP-1β,[70] which have all been mapped to human chromosome 17q11.2-q21. Boxes denote exons, with open spaces representing untranslated regions, hatched areas representing signal peptide coding nucleotides, and filled areas representing mature protein coding nucleotides. Numbers above boxes denote amino acids encoded by that exon. Lines represent introns, with number in parentheses representing the number of nucleotides in that intron. (B) Comparison of amino acid and nucleotide sequences around intron/exon boundaries in human and mouse molecules. Nucleotides contained within exons are capitalized; nucleotides within introns are in lower case. Amino acids are represented in three-letter code. The number of amino acids (X) present in exon 2 between those listed is denoted by the subscript numeral. HuMIP-1α* represents two characterized genes[16,50,51] with nucleotide and amino acid differences in exon 1, intron 1, and exon 2 as shown.

though typically C-X-C genes contain one additional exon, encoding a few carboxyl-terminal amino acids and the 3' untranslated regions.

The RANTES/SIS cytokine genes are not only related in their structural organization, but they appear to be closely clustered on human chromosome 17. Current data put the location of the RANTES/SIS gene complex at 17 q11-q21.[50,66,70] Irving et al. indicate that genes encoding HuMIP-1α and HuMIP-1β are separated by only 14 kb.[50] A similar situation exists in mice, where all of the mouse homologs of RANTES/SIS cytokines so far mapped appear to be closely clustered on murine chromosome 11.[68] The murine cluster lies on a portion of chromosome 11 so extensively homologous to human chromosome 17 that linkage data from this region of the mouse chromosome has been applied to analyses of the human genome.[68] The C-X-C cytokine genes examined to date are also clustered in humans, and have been mapped to chromosome 4 q21-q23 (for review see reference 1).

Some of the variation seen among the independent clones for HuMIP-1α and especially HuMIP-1β (Table 1 and Fig. 4) may be explained by the presence of multiple, non-allelic forms of the genes encoding these cytokines. There are reports of two functional HuMIP-1α genes with the presence of at least one more HuMIP-1α locus whose transcriptional status is not known,[16,50,51] and Irving et al. show that at least two HuMIP-1β genes exist.[50] It has been proposed that variable amplification events are responsible for the occurrence of the microheterogeneity among HuMIP-1 cytokines and the prevalence of their expression. With HuMIP-1α, one of the genes seems preferentially expressed in adult lymphoid cells[16,50] and all cloned HuMIP-1α cDNAs to date are identical. The number of cloned cDNA variants for HuMIP-1β suggests that one gene is not expressed as preferentially as that seen with HuMIP-1α.

The RANTES/SIS cytokines map to the same region of chromosome 17 that has been implicated in von Reckinghauser neurofibromatosis (NF-1 gene) and in acute promyelocytic leukemia. Given that HuMIP-1α has been shown in the mouse to have an inhibitory effect on some hematopoietic precursor cells,[20] the role of the RANTES/SIS cytokines in proliferative disorders bears further investigation.

Potential Regulatory Elements

Despite predicted structural similarities and the clustering of the genes on human chromosome 17, transcription of the RANTES/SIS cytokine genes is differentially regulated. While most are massively inducible in hematopoietic cells, RANTES is not, and the expression of such genes as I-309 and MCP-1 is widely disparate. Figure 6A shows that in the functional T cell line DLαSH the accumulation of mRNA for both HuMIP-1α and -1β is highly induced within 2 hours after antigen stimulation, and begins to return to basal levels 72 hours after stimulation. In contrast, an inverse pattern of RANTES mRNA expression is detected in the same cells, and MCP-1 is not expressed. In freshly isolated PBMC, HuMIP-1α and -1β are again highly induced shortly after PHA and TPA treatments, but relative levels of RANTES and MCP-1 mRNA remain low. The inducibility and relative levels of mRNA for these cytokines in the T cell line TKαJY and HL-60 cells are compared in Fig. 6B. RANTES mRNA levels are not affected by phorbol myristate acetate treatment of HL-60 cells, but these levels are markedly less than those seen with an equivalent amount of T-cell RNA. In contrast, MCP-1, HuMIP-1α, and HuMIP-1β RNAs are all variably induced in the same cells. MCP-1 is not expressed in the T cell line, and its expression is highly induced from basal levels in HL-60 cells after phorbol ester treatment. HuMIP-1α is induced to approximately the same level in both cell types, and HuMIP-1β is barely detected in the HL-60 cells even though it is highly abundant in the T-cell RNA. These results exemplify the fact that myeloid and lymphoid cells appear capable of regulating these genes differently, and even the tightly linked HuMIP-1α and HuMIP-1β can be independently expressed.

Table 2 lists the tissues and cell types that have been reported to express mRNA for these cytokines in response to various treatments. MCP-1 has the widest expression pattern, while expression of the other members of this family, particularly RANTES and I-309, appears to be more restricted.

There is reason to believe that RANTES/SIS cytokines are differentially expressed in vivo as well. In situ hybridization studies in this laboratory have indicated that while RANTES mRNA expression is widespread throughout the cortex of the thymus, HuMIP-1α and -1β expression in the same section is limited to discrete locations with highly expressing cells. In situ hybridization patterns in the spleen for RANTES and HuMIP-1α, -1β are also distinct.[22]

The regulatory elements controlling the differential expression of these genes, and particularly the promoters which allow for such rapid and substantial induction of MCP-1 in fibroblasts and HuMIP-1α and -1β in lymphocytes, would be of considerable interest. The precise nature of these regulatory elements is not yet defined, but a number of potential controlling elements in the presumed regulatory regions of these genes have been sequenced (Table 2). Many of these sites, particularly those in MCP-1 and I-309, are known to be conserved between rodents and human.[66,71-73] TPA response elements, which are consensus AP1-binding sites, an octamer transcription factor consensus binding site, and a κB enhancer element consensus site
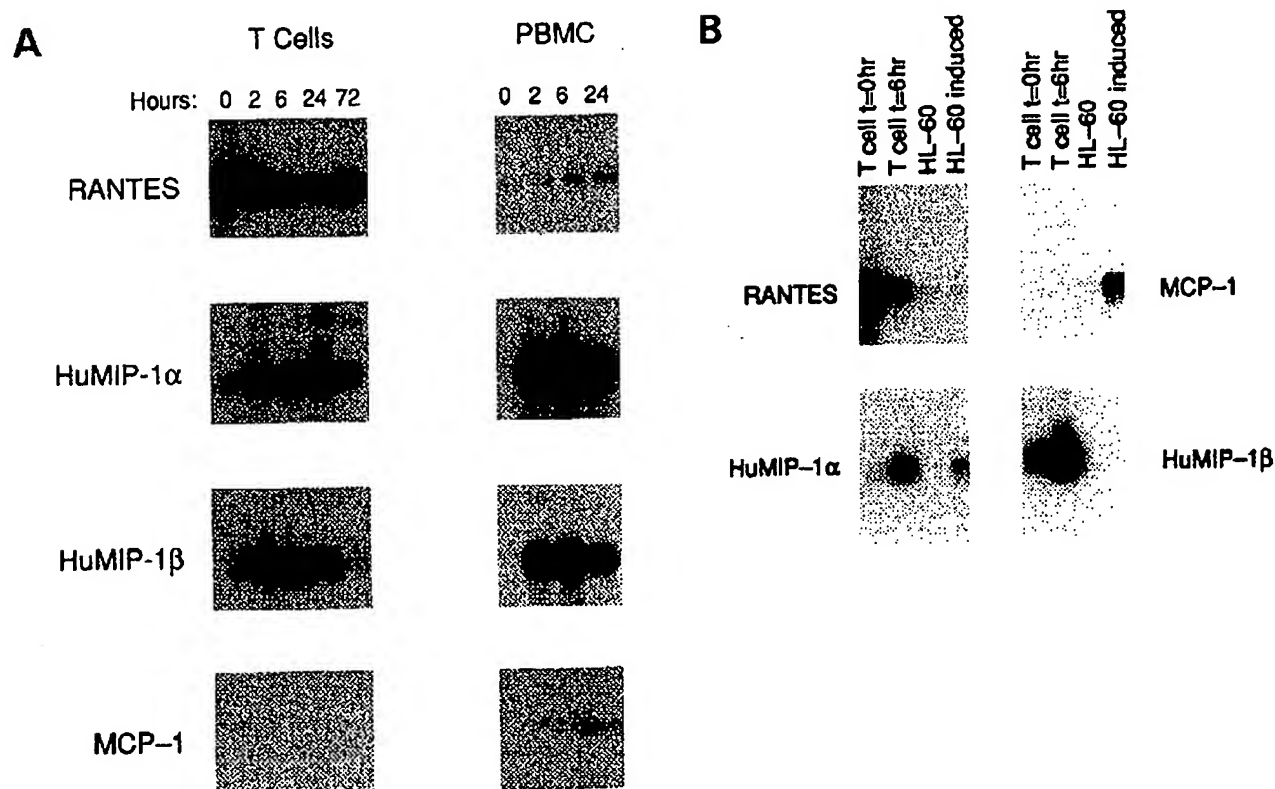
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Figure 6. Northern analysis of RANTES/SIS cytokine mRNA.

(A) RANTES, HuMIP-1α, HuMIP-1β, and MCP-1 mRNA levels in the cultured functional cytotoxic T cell line DLαSH before and after antigen stimulation (left panel). Gel contains 7.5 μg per lane of total RNA isolated from cells prior to stimulation with alloantigen (t = 0 h) and 2, 6, 24, and 72 h after stimulation. The same blot was stripped and reprobed with a labeled cDNA representing the coding region for each cytokine. Right panel shows levels of cytokine mRNA in fresh peripheral blood mononuclear cells before and after mitogen stimulation. The blot was prepared as above from PBMC stimulated at t = 0 with phorbol ester and PHA, with time points collected at 2, 4, and 24 h after stimulation. (B) RNA gel blot containing 7.5 μg per lane of total RNA isolated from the functional cell line TK α JY prior to stimulation with alloantigen (t = 0) and 6 h after antigen stimulation (t = 6 h) and from the promyelocytic leukemia cell line HL-60 before and after induction with phorbol myristate acetate (50 ng/ml, HL-60 induced).

have all been detected in the 5' flanking region of the human MCP-1 gene.[71,72] In addition, the rat JE gene promoter has been shown to contain an identical AP1 site, which binds a cellular factor in response to serum induction.[73] High levels of mRNA expression are not solely a function of gene transcription, however: increased accumulation of JE mRNA in macrophages is reported to be due to increased mRNA stability,[74] and the increase of JE mRNA in response to IFN-γ in macrophages appears to be a function of cytosolic alkalinization.[75]

RECEPTORS

Recent efforts have sought to characterize the RANTES/SIS cytokine receptors, and, though no molecular structures have been published to date, some useful data is emerging. Yoshimura and Leonard report the characterization of MCP-1 binding sites on

monocytes with an affinity of $\sim 2.0 \times 10^{-9}$ M, and 1,200 to 3,400 sites/cell.[46] No significant binding to lymphocytes or to a variety of leukemia or lymphoma cell lines was seen, nor was binding to neutrophils detected.[46] This is consistent with the fact the MCP-1 is a potent attractant for monocytes but has no effect on neutrophils. Using protein purified from a fibrosarcoma cell line, however, Matsushima and colleagues report that bound MCP-1 is rapidly internalized and that this internalization cannot be inhibited by standard biochemical methods. The number of binding sites or the binding affinity of the MCP-1 receptor was therefore impossible to ascertain in that study.[76]

Act-2, one of the HuMIP-1β variants, binds to sites on PHA/PMA-stimulated PBMC, MT-2 cells, HL-60, K562, and HeLa cells.[77] Napolitano et al. report a K_d of 7.8×10^{-9} to 12×10^{-9} M, with between 7,000 and 10,000 receptors/cell and as many as 45,000 receptors/cell on activated PBMC. Binding was not detected with

Table 2. Potential regulatory sequences and distribution of expression of RANTES/SIS cytokines.

Potential regulatory elements controlling RANTES/SIS cytokine gene transcription and distribution of RANTES/SIS mRNA expression (both human and mouse). References are listed in parentheses.

Cytokine	Possible regulatory element	Tissues or cells capable of expressing mRNA (including species homologs)
MCP-1	TPA-responsive elements (TRE) (AP1-binding site) Octamer transcription factor binding site NF-κB enhancer element Interferon consensus sequence Lymphokine decanucleotide core sequence (71-73)	Fibroblasts Glioma Endothelial cells Monocytes Vascular smooth muscle Various non-hematopoietic tumor cells PC12 (rat nerve) cells Pigmented retinal epothelial cells Epidermal keratinocytes
I-309	NF-κB binding site EIA enhancer core PU-1 transcription factor binding site Py early consensus sequence Ig octamer Lymphokine decanucleotide core sequence (66,68)	T cells Monocytes Mast cells
RANTES		T cells HL-60 RD, MG63 (rhabdomyosarcoma, osteosarcoma)
HuMIP-1α	NF-κB–binding site HIV-1 enhancer Transregulation core sequence CK-2 transcription factor binding site SRE core sequence GASNE negative regulatory element motif Lymphokine decanucleotide consensus sequence Cytokine promoter sequence (16,50,51,69)	T cells B cells Monocytic cells U937 HL-60 Fibroblasts Glioma HTLV-1–infected T cells (constitutive) Mast cells
HuMIP-1β	HTLV-1 tax responsive AP1-binding site (91)	T cells B cells Monocytic cells Mast cells U937 cells
HC14		INF-γ–stimulated monocytes

unstimulated PBMC. There has also been a preliminary report on the binding of HuMIP-1α to U937 cells.[78]

Data from studies with IL-8 and MCP-1 suggest that IL-8 binds to and attracts neutrophils while MCP-1 binds to and attracts monocytes. At least one other C-C cytokine, RANTES, attracts monocytes but not neutrophils.[67] This raises the possibility that C-X-C molecules might generally affect neutrophils while C-C (RANTES/SIS) cytokines affect monocytes. Observations made in this laboratory (unpublished data), that recombinant RANTES/SIS cytokines will mobilize calcium in monocytes but not in neutrophils, are consistent with this model. It has been reported, however, that murine MIP-1α, -1β, and TCA3 will promote neutrophil infiltration in animal studies.[18,65] It has not been shown that this is a direct effect, however, and there are no published reports that the human RANTES/SIS cytokines have any attractive effects on neutrophils either in vitro or in vivo. Since the RANTES/SIS cytokines appear to have attractive effects on distinct populations of lymphocytes (see below), it might be expected that each will have its own unique cell surface receptor. Alternatively, there may be associated accessory binding or signaling proteins that confer cytokine-specific effects in different cell types.

Even if the cellular receptors for IL-8 and MCP-1 are distinct, however, other common binding structures may exist for these molecules. Baker and colleagues have observed displaceable high-affinity binding of IL-8 and MCP-1 to the surface of erythrocytes. Moreover, it appears that IL-8 and MCP-1 compete for the same high-affinity sites on these cells.[79] It has been postulated that red cells may act as a physiological "sink" for these chemotactic substances in the blood.[79] This mechanism could provide for the sequestering of chemotactic compounds in the blood and prevent their action at inappropriate times or on physiologically inappropriate targets.

Lastly, since it appears the murine MIP-1 proteins effect their febrile actions directly on the hypothalamus,[80] it will be of interest to see if the structures of the brain receptor and immune receptor are similar.

PHYSIOLOGICAL AND PATHOLOGICAL ROLES FOR RANTES/SIS CYTOKINES

Inflammatory Activities

The migration of neutrophils and mononuclear phagocytes to injured tissues is a hallmark of inflammation. Once at the inflamed site, these cells are activated to destroy microorganisms and cellular debris, remove injured and degraded endogenous tissues, and mount an immune response. The complex cascade of inflammatory events is mediated through the various cells near the inflamed site, which are in turn attracted to that site by various chemotactic substances including bacterial membrane components, complement protein fragments, and lymphokines.

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The mouse homologs of RANTES/SIS cytokines have been shown to have a variety of proinflammatory activities. In animal studies the murine MIP-1 protein preparation causes: (1) footpad swelling characterized by neutrophil influx in endotoxin-resistant mice; (2) neutrophil influx followed by a monocytic infiltrate after intracisternal injection in rabbits; and (3) a prostaglandin-independent fever response in rabbits. In vitro, murine MIP-1 is mildly chemotactic for human neutrophils and causes an oxidative burst in these cells at high concentrations (for review see reference 47). Partially purified recombinant TCA3, the murine homolog of I-309, also causes swelling and neutrophil accumulation after injection in mouse footpads.[65] Much less information has been published on purified recombinant proteins, particularly in the human system. MCP-1 has potent monocyte chemotactic activity in vitro, but does not attract neutrophils.[8,36] Injection of MCP-1 into rat ears is followed by a monocytic infiltration after 6 hours with a maximal response after 16 hours.[8] RANTES has also been reported to attract monocytes, but not neutrophils, in vitro, as well as very specific T cell subsets.[67] Little data has been published on the activities of HuMIP-1α and -1β or I-309. Studies in this laboratory with conditioned media containing recombinant HuMIP-1α and -1β proteins fail to detect significant neutrophil attraction and donor to donor variation in monocyte attractive potential (unpublished observations). HuMIP-1α and -1β do attract distinct populations of lymphocytes in in vitro assays (reference 22, and see below).

Much of the in vitro evidence obtained using recombinant human RANTES/SIS cytokines is consistent with the idea that the C-C cytokines in the PF4 superfamily might play a role in chronic inflammation as mediated through monocytes, while C-X-C proteins are involved in acute inflammation as mediated through neutrophils. Clearly further experimentation is needed both in vitro and in animal models with purified recombinant cytokines to address this question.

Lymphocyte Trafficking and Hematopoietic Regulation

Several recent findings suggest that RANTES/SIS cytokines may be important in lymphocyte trafficking and in the regulation of hematopoietic progenitor cells. Trafficking involves the migration of different populations of lymphocytes at the proper time to sites of infection or other antigenic challenge. This process can be thought to contain two main components: homing and migration. Homing involves adhesion, and homing receptor molecules have recently been characterized that mediate the passage of leukocytes as a class from the blood to the tissues via the endothelium. However, chemoattractant substances responsible for the migration of specific subsets of lymphocytes to antigenic sites

have been largely unknown. RANTES appears to selectively attract T cells of the memory/helper phenotype in vitro, without affecting B cells or T cells of other phenotypes (Fig. 7, reference 67). Memory T cells, which can be differentiated from naive T cells based on the type of leukocyte common antigen (CD45) isoform they express, are immunologically experienced cells with enhanced proliferative and cytokine-producing potentials. Memory T cells are thought to be key players in chronic inflammation and a variety of autoimmune diseases. Additionally, studies with HuMIP-1β in vitro show it to preferentially attract naive helper T cells, while HuMIP-1α attracts killer T cells and B lymphocytes in a concentration-dependent fashion.[22] These specific, non-overlapping, attractive functions indicate that RANTES/SIS cytokines can affect most of the major subsets of immune effector cells (Fig. 8), and suggest that they may have key roles in lymphocyte trafficking.

Murine MIP-1 proteins have been shown to be regulators of various hematopoietic progenitor cells. MIP-1 can synergise with GM-CSF and M-CSF to enhance the formation of granulocyte-macrophage colonies by murine granulocyte/macrophage progenitor cells (CFU-GM).[49] In contrast, MIP-1α, but not MIP-1β, is a specific inhibitor of hematopoietic stem cell proliferation.[20] The idea that RANTES/SIS cytokines may have growth- and proliferation-regulating activities is an intriguing one. In another situation
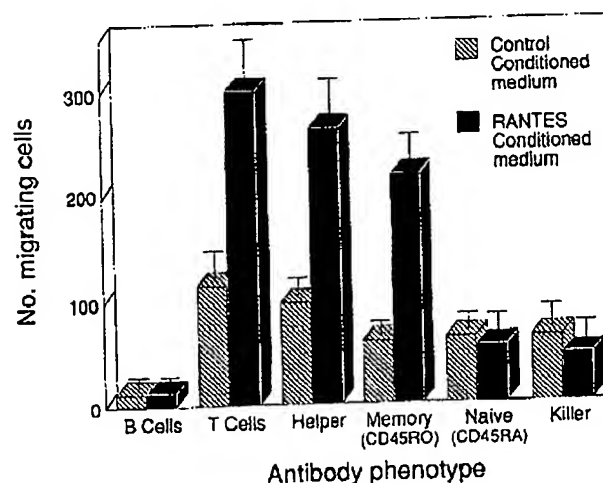
Figure 7. Selective attraction of memory T helper cells in response to RANTES.

Phenotype of fresh peripheral blood lymphocytes (PBL) responding to recombinant RANTES-conditioned medium (solid bars) and control conditioned medium (hatched bars) in n = 6 microchemotaxis chamber experiments. PBL were phenotyped using primary antibodies recognizing B cells (Pan B cell), T cells (Pan T cell), or the T cell subset-specific markers CD4 (helper), CD8 (killer), UCHL1 (memory, CD45RO), or SN130 (naive, CD45RA), as described.[67] Histograms represent mean ± SEM number of positively staining cells that have migrated.
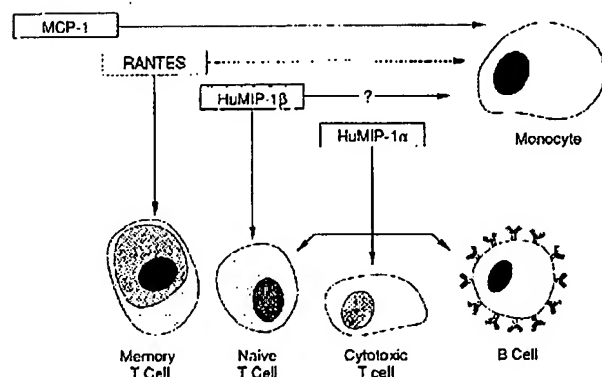
Figure 8. Possible roles for RANTES/SIS cytokines in leukocyte trafficking.

Schematic representation of the attractive potentials of RANTES/SIS cytokines for distinct subpopulations of immune effector cells, based on in vitro chemotaxis assays.

where proliferation is controlled, T cell anergy induction, we have shown that mRNA for HuMIP-1α and HuMIP-1β is superinduced, relative to normal antigen induction, during the development of the non-responsive state.[81]

Roles in Wound Healing

There has been much speculation about the roles PF4 superfamily cytokines in wound healing. Most of these activities have been associated with the C-X-C branch of the superfamily, but a small body of work to date indicates that C-C cytokines might also have roles in this setting. Experiments using wound healing chambers have detected murine MIP-1 cytokines in early wound inflammatory cells, and MIP-1 has been shown to stimulate wound fibroblasts to produce IL-1 and IL-6.[82] Epidermal keratinocytes have been shown to produce MCP-1,[63] and dermal fibroblasts can be induced to express mRNA for MCP-1, HuMIP-1α, and RANTES (reference 51 and unpublished observations). Recombinant RANTES/SIS cytokines have not been found to have keratinocyte chemoattractant properties in vitro (unpublished observations).

RANTES/SIS Cytokines in Tumors

A diverse collection of non-hematopoietic solid tumor cell lines secrete a monocyte chemoattractant protein highly related or identical to MCP-1.[61] It has long been known that monocytes and lymphocytes can make up the majority of the mass of certain solid tumors (for review see reference 83), and it has been suggested that MCP-1 may be responsible for this monocyte component.[61] Interestingly, Graves et al. report that a polyclonal antibody raised against baboon MCP-1 could abrogate 100% of the monocyte chemotactic activity released by many solid tumor cells lines,

including RD, a rhabdomyosarcoma, and MG63, an osteosarcoma.[61] We have noted, however, that both of these cell lines also express RANTES mRNA (Fig. 9). The level of RANTES mRNA expressed in these cells is far less than that expressed in T cells, and less than the level of MCP-1 RNA. Because the anti–MCP-1 antibody neutralizes the monocyte-attracting activities produced by RD and MG-63,[61] it is possible that it cross-reacts with RANTES. Alternatively, RANTES may be transcribed but not synthesized by these cells, or the concentration of RANTES secreted by RD and MG63 may not be within the effective range of monocyte chemoattraction. We do not detect HuMIP-1α or -1β RNA in these cells (Fig. 9). Production of RANTES by solid tumor cells could explain in part the association of lymphocytes with certain tumors.

MCP-1 may activate monocytes to release cytostatic and perhaps cytotoxic activities. Purified MCP-1 was shown to stimulate normal human monocytes to be growth inhibitory in vitro for several human tumor cell lines.[8,30] Moreover, Rollins and Sunday report that tumor formation in vivo is suppressed when malignant cells are engineered to express the MCP-1 gene.[84] Chinese hamster ovary (CHO) or human HeLa cells selected to express varying levels of exogenous MCP-1 do not form tumors when injected into nude mice, while unaltered cells form large tumor masses. The inhibitory effect works in trans—unaltered cells will not form tumors when coinjected with MCP-1–
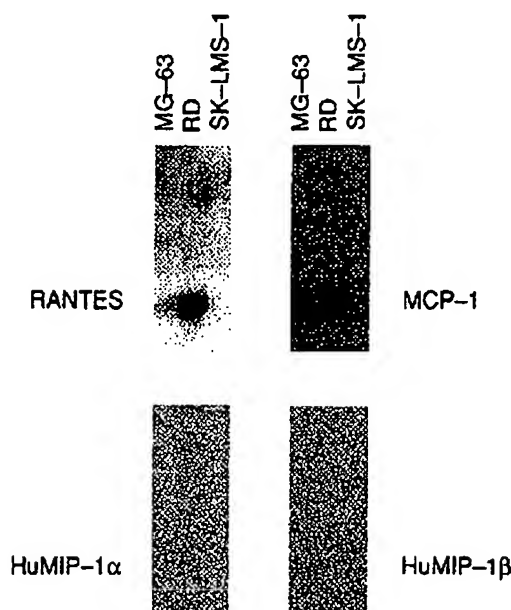
Figure 9. Expression of RANTES/SIS cytokines by non-hematopoietic solid tumor cells.

Northern blot analysis of 50 μg of total RNA from the osteosarcoma line MG63, the rhabdomyosarcoma RD, and the leiomyosarcoma SK-LMS-1. The same blot was stripped and reprobed with a labeled coding-region cDNA for each of the cytokines listed.

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expressing cells—and is also MCP-1 dose-dependent—malignant cells expressing low levels of introduced MCP-1 show tumor suppression for only short periods while cells expressing high levels fail to form tumors even 1 year after injection. Thus, MCP-1 may be involved in host immune surveillance against tumors. Somewhat paradoxically, clinically obtained tumor-associated leukocytes are frequently ineffective at killing host tumors. This, coupled with the observation that many solid tumor cell lines express and secrete MCP-1,[61] raises the possibility that the interaction between tumor and leukocyte is somewhat beneficial to the tumor. One possibility is that tumors might produce MCP-1 and RANTES to derive potential benefits, such as leukocyte-produced angiogenesis factors, or to acquire an insulative cover of inactivated immune cells to provide a shield against more effective host surveillance.

RANTES/SIS Cytokines in Atherosclerosis

Enhanced recruitment of monocytes from the blood to the arterial intima is a feature of atherosclerosis. Foam cells characteristic of early atherosclerotic lesions known as fatty-streaks are thought to be macrophage derived. In vitro studies show that MCP-1 can be secreted by several of the major components of the arterial wall: endothelial cells, fibroblasts, and arterial smooth muscle cells.[36,59,60] MCP-1 produced by any of these cell types could account for the initial attraction of monocytes, resulting in fatty streak formation, subsequent endothelial cell damage, and exposure of the basement membrane. One potential early stimulus of MCP-1 secretion could be minimally modified low density lipoprotein (MM-LDL), which has been shown to induce MCP-1 production in endothelial cells and smooth muscle cells.[85] Once platelets adhere to the exposed basement membrane, PDGF could provide additional MCP-1 induction in fibroblasts. Thus an amplifying loop of MCP-1 production could be set up and might include production of MCP-1 by the attracted monocytes themselves once activated at the site of the growing neointimal plaque.

In situ and in vivo studies are beginning to lend support to some of these ideas. Marmur et al. report that rat aortas subjected to balloon dilation rapidly express high levels of JE protein.[86] The same report shows JE to be a chemoattractant for vascular smooth muscle cells in vitro. Nelkin et al. report in situ detection of MCP-1 RNA in diseased, but not normal, arteries.[87] In human carotid endarterectomy specimens, MCP-1 was detected primarily in macrophage-rich regions underlying the necrotic core of the plaque and in organizing thrombi. Expression was also detected in localized vascular smooth muscle cells but not, surprisingly, in endothelial cells. MCP-1 was also expressed in proliferative neointimal lesions of heart transplant antherosclerosis.

Studies in this laboratory extend these findings to HuMIP-1α, -1β, and RANTES. We detect high levels for each of these mRNAs in situ in normal carotid plaque and in heart transplant atherosclerosis (reference 26 and T. Schall, A. Augustine, and J. Wilcox, in preparation). HuMIP-1α, and -1β are typically coexpressed, with very high levels of mRNA present in a band of macrophages running through the neointima in several specimens, an example of which is shown in Fig. 10. RANTES mRNA is not detected in the same cells expressing HuMIP-1α and -1β, but is highly expressed in lymphocytes and macrophages typically more proximal to the lumen. The patterns of expression seen in some of the lesions examined suggest positive feedback mechanisms for these cytokines and possible differential expression of these cytokines at various stages in the progression of arterial disease.

Possible Roles in Rheumatoid Arthritis

The rheumatoid synovial environment is an area of intense immunological activity, characterized by a proliferation of synovial lining cells and the accumulation of inflammatory cells, including mononuclear cells and lymphocytes. Ultimate destruction of joint tissue is
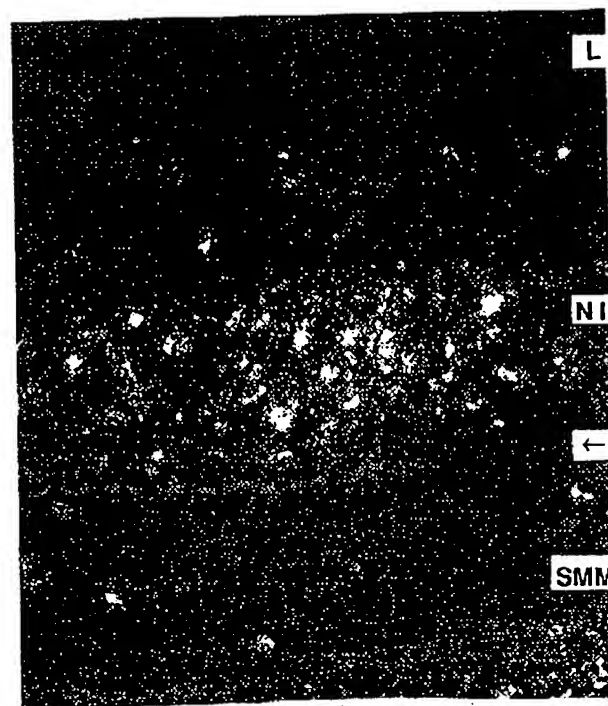

Figure 10. Darkfield polarized-light epiluminescence view of carotid transplant atherosclerotic plaque.

Shown is hybridization (bright grains) for HuMIP-1β in individual cells of the smooth muscle media (SMM) and in a band of cells in the neointimal plaque (NI). Arrow, position of internal elastic lamina; L, lumen. Four-week exposure, 125× magnification.

thought to occur partially as a result of the degradative activities of these inflammatory cells. The substances responsible for the accumulation of leukocytes in the rheumatoid synovial environment have been unknown.

Brennan et al. have reported the presence of IL-8 in rheumatoid synovial fluid and IL-8 mRNA expression by isolated synovial cells.[88] Rathanaswami et al. find very high concentrations of IL-8 released from rheumatoid synovial fibroblasts after stimulation in culture with TNF-α or IL-1.[89] In addition, we have detected the expression of the RANTES/SIS cytokines in fibroblasts cultured from rheumatoid joints. MCP-1, RANTES, and HuMIP-1α all appear to be massively induced and secreted after stimulation of these cells with IL-1 or TNF (unpublished observations). In fact, this is the only setting in which the relatively rapid induction of RANTES mRNA has been detected.

Many of the features of the rheumatoid synovial environment suggest possible roles for RANTES/SIS cytokines. The presence of a large number of infiltrating mononuclear cells and polymorphonuclear leu-

kocytes could be a response to the elaboration of MCP-1, RANTES, and IL-8. Moreover, memory T helper cells seem to be attracted to and retained at the proliferating synovial lining. Since both RANTES and IL-8 have been shown to be attractants for T cells,[67,90] they may play a role in the association of T cells and the synovial lining in rheumatoid disease processes.

SUMMARY AND PERSPECTIVES

The discovery and characterization of the RANTES/SIS cytokines and the PF4 superfamily to which they belong has defined a new area in immunobiology. Clearly much remains to be done in this area. Of particular interest will be the nature of the specific cell surface receptors for these cytokines and their signal transduction mechanisms. In addition, there are at least three areas where understanding the regulation of the expression of these genes would be of interest: (1) elements responsible for the very active transcriptional kinetics of most of these genes in stimulated
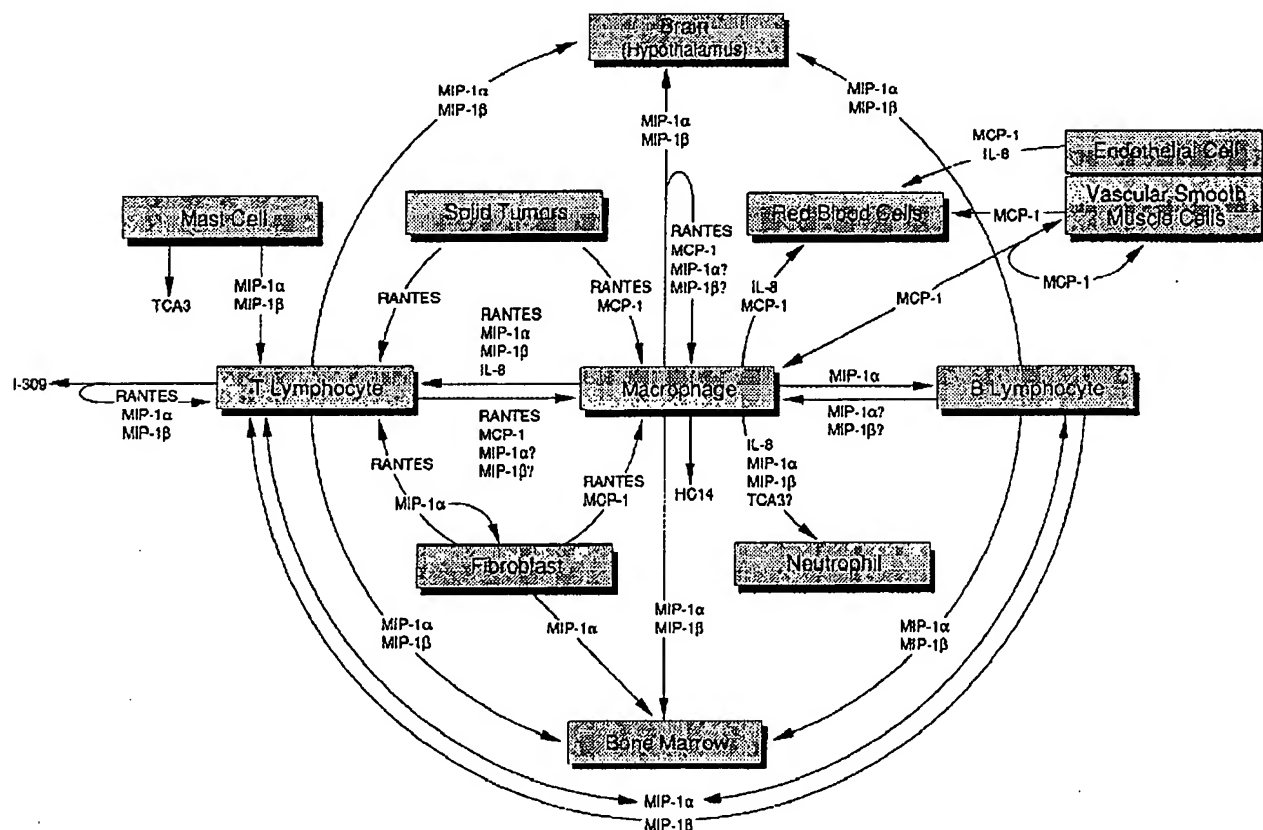
Figure 11.   Possible involvement of RANTES/SIS cytokines in immune and homeostatic regulation.

In this partial listing of the network involving these molecules, arrows pointing *away* denote cells known to express mRNA or secrete protein for the cytokine listed. Arrows pointing *in* indicate cells known to respond to, or to have binding sites for, the cytokines listed. Question marks indicate activities or sites of mRNA expression for which some preliminary evidence exists. Some functions where IL-8 is known to be involved have been added for comparison. The murine MIP designation has been used throughout, and any activity identified for the murine MIP-1 protein preparation is listed here with both proteins, MIP-1α and MIP-1β.

cells; (2) elements responsible for the restricted range of tissue expression; and (3) elements governing the inverse expression patterns of such genes as RANTES and the HuMIP-1 genes in T lymphocytes. The cooperative and synergistic activities of the cytokines in this family bear further investigation, as do the effects of these cytokines on the inhibition and stimulation of various cell types. For example, do the inhibitory properties of MIP-1α on hematopoietic stem cells extend to other cell types, and can other cytokines of this class also affect the proliferative potentials of cells? Moreover, what is the functional significance of the microvariant HuMIP-1 proteins and do variant clones such as P500 and MAD-5α represent alternatively spliced transcripts that encode proteins structurally different from the other RANTES/SIS cytokines?

The body of data reviewed here gives evidence for the involvement of the RANTES/SIS cytokines in an impressive array of immunoregulatory and inflammatory functions and in a number of pathological conditions. These cytokines may act as key links between a variety of cells and tissues, providing common threads between physiological and pathological states. Figure 11 traces some of these links, and highlights the fact that these cytokines are at once diverse in distribution yet specific in function. Monocyte chemoattraction, for example, is an important component of such diverse states as inflammation, the growth of certain solid tumors, and the development of atherosclerotic lesions. These states, varied as they are, could all be mediated in part through the specific action of MCP-1. In like fashion, the extraordinary ability of RANTES to attract specific T cell subsets makes it an attractive candidate for roles in conditions where T cells predominate, including autoimmune disease, certain neoplasms, and chronic inflammatory conditions. Indeed, if the actions of the RANTES/SIS cytokines in vivo mirror those exhibited in vitro, they may mediate essential processes in lymphocyte trafficking through their ability to target distinct subsets of immune effector cells. Further investigation of these molecules and their properties could provide key insights into basic immune function and could lead to advances in the clinical manipulation of autoimmune disorders, cancer, atherosclerosis, and chronic inflammation.

Acknowledgements

Note Added in Proof

A new murine member of the C-C branch of the PF4 superfamily, C10, has been described,[92] and recombinant human I-309 protein has been reported to be a chemoattractant for monocytes but not for neutrophils in vitro.[93]

REFERENCES

1. Stoeckle MY, Barker KA (1990) Two burgeoning families of platelet factor 4-related proteins: mediators of the inflammatory response. New Biol 2:313-323.

2. Haskill S, Peace A, Morris J, Sporn SA, Anisowicz A, Lee SW, Smith T, Martin G, Ralph P, Sager R (1990) Identification of three related human GRO genes encoding cytokine functions. Proc Natl Acad Sci USA 87:7732-7736.

3. Vanguri P, Farber JM (1990) Identification of CRG-2. J Biol Chem 265:15049-15057.

4. Farber JM (1990) A macrophage mRNA selectively induced by γ-interferon encodes a member of the platelet factor 4 family of cytokines. Proc Natl Acad Sci USA 87:5238-5242.

5. Deutsch E, Johnston SA, Seegers WH (1955) Differentiation of certain platelet factors related to blood coagulation. Circ Res 3:110-115.

6. Deuel TF, Keim PS, Farmer M, Heinrikson RL (1977) Amino acid sequence of platelet factor 4. Proc Natl Acad Sci USA 74:2256-2258.

7. Baggiolini M, Walz A, Kunkel SL (1989) Neutrophil activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. J Clin Invest 84:1045-1049.

8. Matsushima K, Oppenheim JJ (1989) Interleukin 8 and MCAF: novel inflammatory cytokines inducible by IL 1 and TNF. Cytokine 1:2-12.

9. Yoshimura T, Matsushima K, Tanaka S, Robinson EA, Appella E, Oppenheim JJ, Leonard EJ (1987) Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. Proc Natl Acad Sci USA 84:9233-9237.

10. Schall TJ, Jongstra J, Dyer BJ, Jorgensen J, Clayberger C, Davis MM, Krensky AM (1988) A human T cell-specific molecule is a member of a new gene family. J Immunol 141:1018-1025.

11. Miller MD, Hata S, Malefyt RDW, Krangel MS (1989) A novel polypeptide secreted by activated human T lymphocytes. J Immunol 143:2907-2916.

12. Burd PR, Freeman GJ, Wilson SD, Berman M, DeKruyff R, Billings PR, Dorf ME (1987) Cloning and characterization of a novel T cell activation gene. J Immunol 139:3126-3131.

13. Brown KD, Zurawski SM, Mosmann TR, Zurawski G (1989) A family of small inducible proteins secreted by leukocytes are members of a new superfamily that includes leukocytes and fibroblast-derived inflammatory agents, growth factors, and indicators of various activation processes. J Immunol 142:679-687.

14. Obaru K, Fukuda M, Maeda S, Shimada K (1986) A cDNA clone used to study mRNA inducible in human tonsillar lymphocytes by a tumor promoter. J Biochem 99:885-894.

15. Zipfel PF, Balke J, Irving SG, Kelly K, Siebenlist U (1989) Mitogenic activation of human T cells induces two closely related genes which share structural similarities with a new family of secreted factors. J Immunol 142:1582-1590.

16. Blum S, Forsdyke RE, Forsdyke DR (1990) Three human homologs of a murine gene encoding an inhibitor of stem cell proliferation. DNA Cell Biol 9:589-602.

17. Zurawski G, Benedik M, Kamb BJ, Abrams JS, Zurawski SM, Lee FD (1986) Activation of mouse T helper cells induces abundant preproenkalphalin mRNA synthesis. Science 232:772-775.

18. Wolpe SD, Davatelis G, Sherry B, Beutler B, Hesse DG, Nguyen HT, Moldawer LL, Nathan CF, Lowry SF, Cerami A (1988) Macrophages secrete a novel heparin-binding protein with inflammatory and neutrophil chemokinetic properties. J Exp Med 167:570-581.

19. Davatelis G, Tekamp-Olson P, Wolpe SD, Hermsen K, Luedke C, Gallegos C, Coit D, Merryweather J, Cerami A (1988) Cloning and characterization of cDNA for murine macrophage inflammatory protein (MIP), a novel monokine with inflammatory and chemokinetic properties. J Exp Med 167:1939-1944.

20. Graham GJ, Wright EG, Hewick R, Wolpe SD, Wilkie NM, Donaldson D, Lorimore S, Pragnell IB (1990) Identification and characterization of an inhibitor of haemopoietic stem cell proliferation. Nature 344:442-445.

21. Kwon BS, Weissman SM (1989) cDNA sequences of two inducible T-cell genes. Proc Natl Acad Sci USA 86:1963-1967.

22. Schall TJ, Bacon K, Camp RDR, Herbert C, Goeddel DV (submitted) Human MIP-1α and MIP-1β cytokines attract distinct populations of lymphocytes.

23. Lipes MA, Napolitano M, Jeang KT, Chang NT, Leonard WJ (1988) Identification, cloning, and characterization of an immune activation gene. Proc Natl Acad Sci USA 85:9704-9708.

24. Chang HC, Reinherz EL (1989) Isolation and characterization of a cDNA encoding a putative cytokine which is induced by stimulation via the CD2 structure on human T lymphocytes. Eur J Immunol 19:1045-1051.

25. Schall TJ, Toy K, Goeddel DV (in press) Biological properties of the RANTES/SIS cytokine family. In Westwick J, and Kunkel S, Lindley IJD (eds) Chemotactic Cytokines: Biology of the Inflammatory Peptide Supergene Family. The Second International Symposium on Chemotactic Cytokines. Humana.

26. Wilcox JN, Augustine AJ, Schall TJ, Gordon D (1990) Local gene expression in human coronary arteries from transplanted hearts analyzed by in situ hybridization. Circulation 82:III 699.

27. Sporn SA, Eierman DF, Johnson CE, Morris J, Martin G, Ladner M, Haskill S (1990) Monocyte adherence results in selective induction of novel genes sharing homology with mediators of inflammation and tissue repair. J Immunol 144:4434-4441.

28. Sherry B, Tekamp-Olson P, Gallegos C, Bauer D, Davatelis G, Wolpe SD, Masiarz A, Coit D, Cerami A (1988) Resolution of the two components of macrophage inflammatory protein 1, and cloning and characterization of one of those components, macrophage inflammatory protein 1 β. J Exp Med 168:2251-2259.

29. Yoshimura T, Yuhki N, Moore SK, Appella E, Lerman MI, Leonard EJ (1989) Human monocyte chemoattractant protein-1 (MCP-1): full-length cDNA cloning, expression in mitogen-stimulated blood mononuclear leukocytes, and sequence similarity to mouse competence gene JE. FEBS Lett 244:487-493.

30. Matsushima K, Larsen CG, DuBois GC, Oppenheim JJ (1989) Purification and characterization of a novel monocyte chemotactic and activating factor produced by a human myelomonocytic cell line. J Exp Med 169:1485-1490.

31. Furutani Y, Normura H, Notake M, Oyamada Y, Fukue T, Yamada M, Larsen CG, Oppenheim JJ, Matsushima K (1989) Cloning and sequencing of the cDNA for human monocyte chemot-

actic and activating factor (MCAF). Biochem Biophys Res Comm 159:249-255.

32. Rollins BJ, Stier P, Ernst T, Wong GG (1989) The human homolog of the JE gene encodes a monocyte secretory protein. Mol Cell Biol 9:4687-4695.

33. Snyderman R, Oppenheim JJ, Mergenhagen SE (1973) A human mononuclear leukocyte chemotactic factor: characterization specificity, and kinetics of production by homologous leukocytes. J Immunol 110:801-810.

34. Robinson EA, Yoshimura T, Leonard EJ, Tanaka S, Griffin PR, Shabanowitz J, Hunt DF, Appella E (1989) Complete amino acid sequence of a human monocyte chemoattractant, a putative mediator of cellular immune reactions. Proc Natl Acad Sci USA 86:1850-1854.

35. Yoshimura T, Robinson EA, Tanaka S, Appella E, Kuratsu J-I, Leonard EJ (1989) Purification and amino acid analysis of two human glioma-derived monocyte chemoattractants. J Exp Med 169:1449-1459.

36. Leonard EJ, Yoshimura T (1990) Human monocyte chemoattractant protein-1 (MCP-1). Immunol Today 11:97-101.

37. Chang HC, Hsu F, Freeman GJ, Griffin JD, Reinherz EL (1989) Cloning and expression of a γ-interferon-inducible gene in monocytes: a new member of a cytokine gene family. Int Immunol 1:388-399.

38. Lee TH, Lee GW, Ziff EB, Vilcek J (1990) Isolation and sequence of eight tumor necrosis factor-induced gene sequences from human fibroblasts. Mol Cell Biol 10:1982-1988.

39. Rollins BJ, Morrison ED, Stiles CD (1988) Cloning and expression of JE, a gene inducible by platelet-derived growth factor and whose product has cytokine-like properties. Proc Natl Acad Sci USA 85:3738-3742.

40. Kawahara RS, Deuel TF (1989) Platelet-derived growth factor-inducible gene JE is a member of a family of small inducible genes related to platelet factor 4. J Biol Chem 264:679-682.

41. Begg GS, Pepper DS, Chesterman CN, Morgan FG (1978) Complete covalent structure of human β-thromboglobulin. Biochemistry 17:1739-1744.

42. St Charles R, Waltz DA, Edwards BFP (1989) The three-dimensional structure of bovine platelet factor 4 at 3.0-Angstrom resolution. J Biol Chem 264:2092-2098.

43. Gregory H, Young J, Schroder J-M, Mrowietz U, Christophers E (1988) Structure determination of a human lymphocyte derived neutrophil activating peptide (LYNAP). Biochem Biophys Res Comm 151:883-890.

44. Tanaka S, Robinson EA, Yoshimura T, Matsushima K, Leonard EJ, Appella E (1988) Synthesis and biological characterization of monocyte-derived neutrophil chemotactic factor. FEBS Lett 236:467-470.

45. Samanta AK, Oppenheim JJ, Matsushima K (1989) Identification and characterization of specific receptors for monocyte-derived neutrophil chemotactic factor (MDNCF) on human neutrophils. J Exp Med 169:1185-1189.

46. Yoshimura T, Leonard EJ (1990) Identification of high-affinity receptors for human monocyte chemoattractant protein-1 on human monocytes. J Immunol 145:292-297.

47. Wolpe SD, Cerami A (1989) Macrophage inflammatory proteins 1 and 2: members of a novel superfamily of cytokines. FASEB J 3:2565-2573.

48. Davatelis G, Wolpe SD, Sherry B, Dayer J-M, Chicheportiche R, Cerami A (1989) Macrophage inflammatory protein-1: a prostaglandin-independent endogenous pyrogen. Science 243:1066-1068.

49. Broxmeyer HE, Sherry B, Lu L, Cooper S, Carow C, Wolpe SD, Cerami A (1989) Myelopoietic enhancing effects of murine macrophage inflammatory proteins 1 and 2 on colony formation in vitro by murine and human bone marrow granulocyte/macrophage progenitor cells. J Exp Med 170:1583-1594.

50. Irving SG, Zipfel PF, Balke J, McBride OW, Morton CC,

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Burd PR, Siebenlist U, Kelly K (1990) Two inflammatory mediator cytokine genes are closely linked and variably amplified on chromosome 17q. Nucleic Acids Res 18:3261-3270.

51. Nakao M, Nomiyama H, Shimada K (1990) Structures of human genes coding for cytokine LD78 and their expression. Mol Cell Biol 10:3646-3658.

52. Kwon BS, Kestler DP, Eshhar Z, Oh K-O, Wakulchik M (1989) Expression characteristics of two potential T cell mediator genes. Cell Immunol 121:414-422.

53. Burd PR, Rogers HW, Gordon JR, Martin CA, Jayaraman S, Wilson SD, Dvork AM, Galli SJ, Dorf ME (1989) Interleukin 3-dependent and -independent mast cells stimulated with IgE and antigen express multiple cytokines. J Exp Med 170:245-257.

54. Yamamura Y, Hattori T, Obaru K, Sakai K, Asou N, Takatsuki K, Ohmoto Y, Nomiyama H, Shimada K (1990) Synthesis of a novel cytokine and its gene (LD78) expressions in hematopoietic fresh tumor cells and cell lines. J Clin Invest 84:1707-1712.

55. Ward PA, Remold HG, David JR (1970) The production by antigen-stimulated lymphocytes of a leukotactic factor distinct from migration inhibitory factor. Cell Immunol 1:167:174.

56. Cochran BH, Reffel AC, Stiles CD (1983) Molecular cloning of gene sequences regulated by platelet-derived growth factor. Cell 33:939-947.

57. Jiang Y, Valente AJ, Williamson MJ, Zhang L, Graves DT (1990) Post-translational modification of monocyte-specific chemoattractant synthesized by glioma, osteosarcoma, and vascular smooth muscle cell. J Biol Chem 265:18318-18321.

58. Decock B, Conings R, Lenaerts JP, Billiau A, Van Damme J (1990) Identification of the monocyte chemotactic protein from human osteosarcoma cells and monocytes: detection of a novel N-terminally processed form. Biochem Biophys Res Comm 167:904-909.

59. Sica A, Wang JM, Colotta F, Dejana E, Mantovani A, Oppenheim JJ, Larsen CG, Zachariae COC, Matsushima K (1990) Monocyte chemotactic and activating factor gene expression induced in endothelial cells by IL-1 and tumor necrosis factor. J Immunol 144:3034-3038.

60. Yoshimura T, Leonard EJ (1990) Secretion by human fibroblasts of monocyte chemoattractant protein-1, the product of gene JE. J Immunol 144:2377-2383.

61. Graves DT, Jiang YL, Williamson MJ, Valente AJ (1989) Identification of monocyte chemotactic activity produced by malignant cells. Science 245:1490-1493.

62. Elner VM, Strieter RM, Elner SG, Kunkel S (1990) MCP gene expression by cytokine treated human retinal pigment epithelium cells. FASEB J 4:A641.

63. Barker JNWN, Jones ML, Mitra RS, Swenson C, Johnson K, Fantone JC, Dixit VM, Nickoloff BJ (in press) Monocytic chemotaxis and activating factor production by cytokine-activated human epidermal keratinocytes. In Westwick J, Kunkel S, Lindley IJD (eds) Chemotactic Cytokines: Biology of the Inflammatory Peptide Supergene Family. The Second International Symposium on Chemotactic Cytokines. Humana.

64. Valente AJ, Graves DT, Vialle-Valentin CE, Delgado R, Schwartz CJ (1988) Purification of a monocyte chemotactic factor secreted by nonhuman primate vascular cells in culture. Biochemistry 27:4162-4168.

65. Wilson SD, Kuchroo VK, Israel DI, Dorf ME (1990) Expression and characterization of TCA3: a murine inflammatory protein. J Immunol 145:2745-2750.

66. Miller MD, Wilson SD, Dorf ME, Seuanez HN, O'Brien SJ, Krangel MS (1990) Sequence and chromosomal location of the I-309 gene: relationship to genes encoding a family of inflammatory cytokines. J Immunol 145:2737-2744.

67. Schall TJ, Bacon K, Toy KJ, Goeddel DV (1990) Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. Nature 347:669-671.

68. Wilson SD, Billings PR, D'Eustachio P, Fournier REK, Geissler E, Lalley PA, Burd PR, Housman DE, Taylor BA, Dorf ME

(1990) Clustering of cytokine genes on mouse chromosome 11. J Exp Med 171:1301-1314.

69. Grove M, Lowe S, Graham G, Pragnell I, Plumb M (1990) Sequence of the murine haemopoietic stem cell inhibitor/macrophage inflammatory protein 1α gene. Nucleic Acids Res 18:5561.

70. Donlon TA, Krensky AM, Wallace MR, Collins FS, Lovett M, Clayberger C (1989) Localization of a human T-cell-specific gene, RANTES (D17S136E), to chromosome 17 q11.2-q12. Genomics 6:548-556.

71. Mehrabian M, Koprivnikar K, Lusis AL, Fogelman AM (1990) Functional and genetic homology between mouse competence factor JE and human monocyte chemotactic protein MCP-1. Circulation 82:III 93.

72. Shyy YJ, Li YS, Kolattukudy PE (1990) Structure of human monocyte chemotactic protein gene and its regulation by TPA. Biochem Biophys Res Comm 169:346-351.

73. Timmers HTM, Pronk GJ, Bos JL, van der Eb AJ (1990) Analysis of the rat JE gene promoter identifies an AP-1 binding site essential for basal expression but not for TPA induction. Nucleic Acids Res 18:23-34.

74. Koerner TJ, Hamilton TA, Introna M, Tannenbaum CS, Bast RC, Adams DO (1987) The early competence genes JE and KC are differentially regulated in murine peritoneal macrophages in response to lipopolysaccharide. Biochem Biophys Res Comm 149:969-974.

75. Prpic V, Yu SF, Figueiredo F, Hollenbach PW, Gawdi G, Herman B, Uhing RJ, Adams DO (1989) Role of Na⁺/H⁺ exchange by interferon-gamma in enhanced expression of JE and I-Aβ genes. Science 244:469-471.

76. Zachariae COC, Anderson AO, Thompson HL, Appella E, Mantovani A, Oppenheim JJ, Matsushima K (1990) Properties of monocyte chemotactic and activating factor (MCAF) purified from a human fibrosarcoma cell line. J Exp Med 171:2177-2182.

77. Napolitano M, Seamon KB, Leonard WJ (1990) Identification of cell surface receptors for the Act-2 cytokine. J Exp Med 172:285-289.

78. Hattori T, Yamamura Y, Ohmoto Y, Nishida T, Takatsuki K (in press) Synthesis of LD78 protein and its binding to myelomonocytic leukemia cell lines. In Westwick J, Kunkel S, Lindley IJD (eds) Chemotactic Cytokines: Biology of the Inflammatory Peptide Supergene Family. The Second International Symposium on Chemotactic Cytokines. Humana.

79. Darbonne WC, Rice GC, Mohler MA, Apple T, Hebert CA, Valente AJ, Baker J (submitted) Red blood cells are a sink for interleukin 8, a leukocyte chemotaxin.

80. Minano FJ, Sansibrian M, Vizcaino M, Paez X, Davatelis G, Fahey T, Sherry B, Cerami A, Myers RD (1990) Macrophage inflammatory protein-1: unique action on the hypothalamus to evoke fever. Brain Res Bull 24:849-852.

81. Schall TJ, O'Hehir RA, Goeddel DV, Lamb J (submitted) Expression of IL8/NAP-1 and RANTES/SIS cytokines during T cell anergy induction.

82. Fahey TJ III, Sherry B, Tracey KJ, Van Deventer S, Jones WG II, Minei JP, Morgello S, Shires GT, Cerami A (1990) Cytokine production in a model of wound healing: the appearance of MIP-1, MIP-2, cachectin (TNF), and IL-1. Cytokine 2:92-99.

83. Mantovani A (1990) Tumor-associated macrophages. Curr Opin Immunol 2:689-692.

84. Rollins BJ, Sunday ME (in press) Suppression of tumor formation in vivo by expression of the JE gene in malignant cells. Mol Cell Biol.

85. Cushing SD, Berliner JA, Valente AJ, Territo MC, Navab M, Parhami F, Gerrity R, Schwartz CJ, Fogelman AM (1990) Minimally modified low density lipoprotein induces monocytic chemotactic protein 1 in human endothelial cells and smooth muscle cells. Proc Natl Acad Sci USA 87:53134-5138.

86. Marmur JD, Friedrich VL Jr, Rossikhina M, Rollins BJ, Taubman MB (1990) The JE gene encodes a smooth muscle

chemotactic factor that is induced by vascular injury. Circulation 82:III 698.

87. Nelken N, Coughlin S, Gordon D, Wilcox JN (submitted) Monocyte chemoattractant protein-1 in human atheromatous plaques.

88. Brennan FM, Zachariae COC, Chantry D, Larsen CG, Turner M, Maini RN, Matsushima K, Feldmann M (1990) Detection of interleukin 8 biological activity in synovial fluids from patients with rheumatoid arthritis and production of interleukin 8 mRNA by isolated synovial cells. Eur J Immunol 202:2141-2144.

89. Rathanaswami P, Hachicha M, Wong WL, Schall TJ, McColl SR (submitted) Tumor necrosis factor α stimulates expression of the the human interleukin 8 gene in synovial fibroblasts.

90. Larsen CG, Anderson AO, Appella E, Oppenheim JJ, Matsushima K (1989) The neutrophil-activating protein (NAP-1) is also chemotactic for T lymphocytes. Science 243:1464-1466.

91. Napolitano M, Modi WS, Cevario SJ, Seuanez HN, Leonard WJ (1990) The gene encoding the Act-2 cytokine chromosomal localization and identification of 5' regulatory sequences. Clin Res 38:302A.

92. Orlofsky A, Berger MS, Prystowsky MB (1991) GM-CSF regulates a novel member of the MIP-1 cytokine family. J Cell Biochem Suppl 15F:138.

93. Miller MD, Krangel MS (1991) The human I-309 gene encodes a monocyte chemotactic factor. J Cell Biochem Suppl 15F:98.

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